

# Mode of Inhibition of $\beta$ -Hydroxydecanoyl Thioester Dehydrase by 3-Decynoyl-*N*-acetylcysteamine\*

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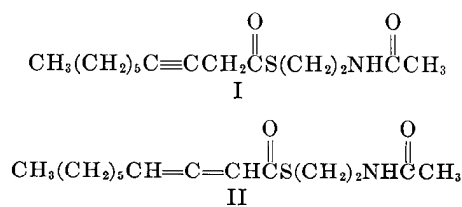
## SUMMARY

The alkynic substrate analogue 3-decynoyl-*N*-acetylcysteamine (NAC) was previously shown to inhibit  $\beta$ -hydroxydecanoyl thioester dehydrase irreversibly. 2,3-Decadienoyl-NAC prepared by chemical isomerization of 3-decynoyl-NAC inhibits dehydrase activity even more effectively. The allenic and the acetylenic inhibitors both inactivate dehydrase by combining with a histidine residue at the active site.  $\alpha$ -Dideutero-3-decynoyl-NAC reacts more slowly with dehydrase than its hydrogen analogue:  $k_H:k_D = 2.60$ . No kinetic isotope effect is observed for the inhibition of enzyme by  $\alpha$ -D-2,3-decadienoyl-NAC. It is concluded that 3-decynoyl-NAC as such is not the true inhibitor, but that the dehydrase isomerizes it to the allene which in turn inactivates the enzyme by a chemical reaction. The finding that free 2,3-decadienoic acid inhibits dehydrase, whereas 3-decynoic acid does not, supports the postulated mode of action.

The *Escherichia coli* enzyme  $\beta$ -hydroxydecanoyl thioester dehydrase catalyzes the reversible interconversions of  $\beta$ -hydroxydecanoyl, *trans*-2-decenoyl, and *cis*-3-decenoyl thioesters (1). Because of its multiple activities dehydrase is considered to be a multifunctional catalyst. The enzyme consists of two identical polypeptide chains of 18,000 mol wt, each of which provides a catalytic site (2).

The alkynic substrate analogue 3-decynoyl-*N*-acetylcysteamine (I) potently inhibits all dehydrase-catalyzed reactions, combining stoichiometrically with the protein and inactivating it by modification of a histidyl residue at each active site (2). This apparently covalent interaction gives rise to a noncompetitive mode of inhibition. Structural features mandatory for inhibitory activity include an acyl chain length of 9, 10, or 11 carbon atoms, a  $\beta,\gamma$ -positioned triple bond, and the presence of

a thioester function (3). 3-Decynoic acid or its methyl ester does not impair dehydrase activity, even at substantially higher concentrations. Since 3-alkynoic acids and their derivatives isomerize under selected conditions to the corresponding allenes (4), the possibility of an acetylene-allene transformation in connection with enzyme inhibition by I was considered. Accordingly, 2,3-decadienoic acid and its NAC<sup>1</sup> ester (II) were prepared and their effects on the dehydrase-catalyzed reactions were tested.



## EXPERIMENTAL METHODS

**2,3-Decadienoic Acid**—3-Decynoic acid (500 mg) was dissolved in 40 ml of 18% aqueous potassium carbonate and the solution was heated at 90° for 5 hours (5). Crystallization of the crude product removed a large portion of the starting material; the combined mother liquors were concentrated and chromatographed on silicic acid (Unisil, Clarkson Chemical Company) with 5 to 10% ether in pentane. Rechromatography yielded the desired compound, which was recrystallized from pentane to give a colorless material (83 mg, 17% of theory), m.p. 24–25°; infrared:  $\nu_{\max}$  3.40, 5.10, 5.95, 6.98, 7.83, and 8.35  $\mu$ . The intensity of absorption at 5.10  $\mu$ , characteristic of allenes, served as a criterion of purity. Nuclear magnetic resonance:  $\delta$ 0.89 (3H, triplet,  $J = 7$  cps), 1.07 to 1.80 (8H, multiplet), 1.9 to 2.3 (2H, multiplet,  $-\text{C}=\text{C}-\text{CH}_2-$ ), 5.49 (1H, doublet,  $J = 6$  cps,  $-\text{C}=\text{CH}-\text{CO}$ ), 5.58 (1H, quartet,  $J = 6$  and 14 cps,  $-\text{CH}_2-\text{CH}=\text{C}=\text{C}$ ), and 12.10 (1H, singlet).

**2,3-Decadienoyl-NAC**—The thioester was prepared by heating 2,3-decadienoyl chloride (6) with a benzene suspension of lead *N*-acetylcysteamine (7) for 2 hours at room temperature. The product was purified by extensive thin layer chromatography. Infrared:  $\nu_{\max}$  2.90, 3.39, 3.47, 5.14, 5.95, and 6.69  $\mu$ . Ultraviolet:  $\lambda_{\max}$  263 nm,  $\epsilon$  2900 (diethyl ether).

Alternatively, and more conveniently,  $10^{-2}$  M 3-decynoyl-NAC was isomerized to 2,3-decadienoyl-NAC by treatment with sodium ethoxide in ethanol at room temperature. At a molar ratio of thioester to ethoxide of 30, the ultraviolet spectrum of

<sup>1</sup> The abbreviation used is: NAC, *N*-acetylcysteamine.

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the thioester underwent a rapid change with disappearance of absorption at 232 nm and appearance of a peak at 263 nm. Exposure to ethoxide for more than 15 min was avoided in order to minimize side reactions. Solvent was removed and

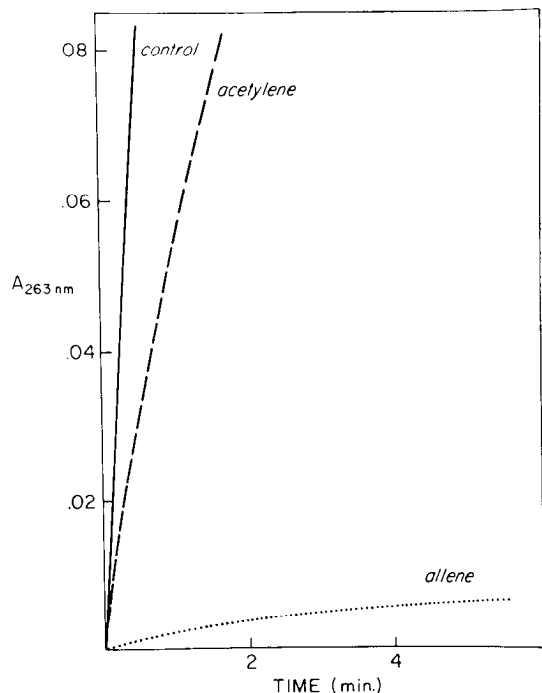


FIG. 1. Comparison of 3-decynoyl-NAC and 2,3-decadienoyl-NAC as dehydrase inhibitors. To cuvettes containing  $10^{-6}$  M thioester inhibitors and  $4 \times 10^{-4}$  M *cis*-3-decynoyl-NAC in 0.01 M potassium phosphate, pH 7.0, were added 6  $\mu$ g of purified dehydrase at zero time. The rate of *trans*-2-decynoyl-NAC production was measured spectrophotometrically.

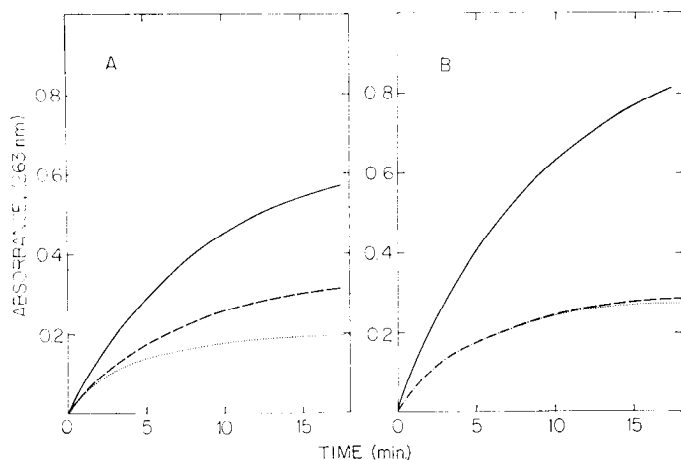


FIG. 2. Effects of deuterium substitution at the  $\alpha$  positions of dehydrase inhibitors. Pure acetylenic and allenic thioesters were dissolved in potassium phosphate, pH 6.0, ionic strength = 0.05, and diluted to known concentrations as determined by their ultraviolet spectra. In each experiment, 33  $\mu$ g of partially purified dehydrase were added at zero time. A, the curves correspond to reaction cuvettes containing, in addition to  $1.65 \times 10^{-4}$  M *cis*-3-decynoyl-NAC: no addition (—),  $5.0 \times 10^{-6}$  M 3-decynoyl-NAC (·····), or  $5.0 \times 10^{-6}$  M 2,2-dideutero-3-decynoyl-NAC (---). B, the curves correspond to reaction mixtures containing, in addition to  $2.18 \times 10^{-4}$  M *cis*-3-decynoyl-NAC: no addition (—),  $1.2 \times 10^{-7}$  M 2,3-decadienoyl-NAC (·····), or  $1.2 \times 10^{-7}$  M 2-deutero-2,3-decadienoyl-NAC (---).

the product purified by silicic acid chromatography with 1% methanol in methylene chloride as the solvent. The yield of allenic thioester was 74%. Ultraviolet:  $\lambda_{\max}$  217 nm,  $\epsilon$  9500; 263 nm,  $\epsilon$  3350 (ethanol).

**2,2-Dideutero-3-decynoyl-NAC**—The deuterated acetylenic acid was synthesized by reaction of 1-octynyl magnesium bromide with ethylene- $d_4$ -oxide (Mallinckrodt/Nuclear, 98% isotopic purity) and oxidation of the resulting alcohol (7). Recrystallization gave 2,2-dideutero-3-decynoic acid in 14% yield: m.p. 35.3–36.0° (reported melting point for 3-decynoic acid, 35.5–38.0° (8)). Infrared:  $\nu_{\max}$  3.40, 4.40, 4.68, 5.80, 7.15, 7.83, 8.48  $\mu$ . The 4.68  $\mu$  absorption is assigned to the C—D stretching vibration. Nuclear magnetic resonance:  $\delta$ 0.88 (3H, triplet,  $J = 5$  cps), 1.33 (8H, multiplet), and 2.13 (2H, triplet,  $J = 6$  cps, —C—CH<sub>2</sub>—C $\equiv$ ).

Thioesterification was achieved by the lead salt method described above. Crystallization from hexane containing a trace of methanol afforded 2,2-dideutero-3-decynoyl-NAC, m.p. 37.0–38.2° (3-decynoyl-NAC, m.p. 38.0–38.8°). Infrared:  $\nu_{\max}$  2.90, 3.39, 3.47, 4.38, 4.67, 5.95, 6.69, and 7.95  $\mu$ . Ultraviolet:  $\lambda_{\max}$  232 nm,  $\epsilon$  4000 (water).

Mass spectral analysis of the thioester confirmed the presence of deuterium in the molecule. Comparison of the deuterated and nondeuterated compounds revealed at least 10 fragments between  $m/e$  81 and 254 differing by 2 mass units. From these data the isotopic composition was calculated to be 62% DD, 30% HD, and 8% HH.

**2-Deutero-2,3-decadienoyl-NAC**—Treatment of 2,2-dideutero-3-decynoyl-NAC with sodium ethoxide in ethyl alcohol- $d$  (Diaprep, Inc.), under the conditions described above for isomerization, led to formation of the desired  $\alpha$ -deuterated allenic thioester. No attempt was made to analyze the absolute deuterium content of the compound.

**Other Methods**—The synthesis of other acyl thioesters has been described (1, 7). Likewise, procedures for enzyme purification, activity measurements, and amino acid analyses have been reported (2, 3, 7). Ultraviolet spectra were recorded with a Unicam SP-800 dual beam instrument, while fixed wavelength measurements were made with a Gilford 240 single beam spectrophotometer. A Perkin-Elmer Infracord spectrophotometer was used to analyze infrared absorptions: nuclear magnetic resonances were detected with Varian A-60 or T-60 instruments. Mass spectra were obtained with an Associated Electrical Industries MS-9 double-focussing instrument.

## RESULTS AND DISCUSSION

As an inhibitor of dehydrase 2,3-decadienoyl-NAC far surpassed the activity of the already powerful 3-decynoyl-NAC. At equal concentrations the allenic isomer diminished enzyme activity at a considerably faster rate than the acetylene (Fig. 1). Furthermore, inhibition could not be relieved by increasing substrate concentration and therefore appeared to be of the same type as the action of acetylenic thioester on dehydrase.

As a more potent enzyme inhibitor than the acetylene, 2,3-decadienoyl-NAC may be assumed to react with dehydrase in a more direct fashion. The question arises whether the allenic and acetylenic inhibitors inactivate dehydrase by different chemical mechanisms or whether the action of the two is re-

<sup>2</sup> Previously, this compound had not been obtained in crystalline form (1).

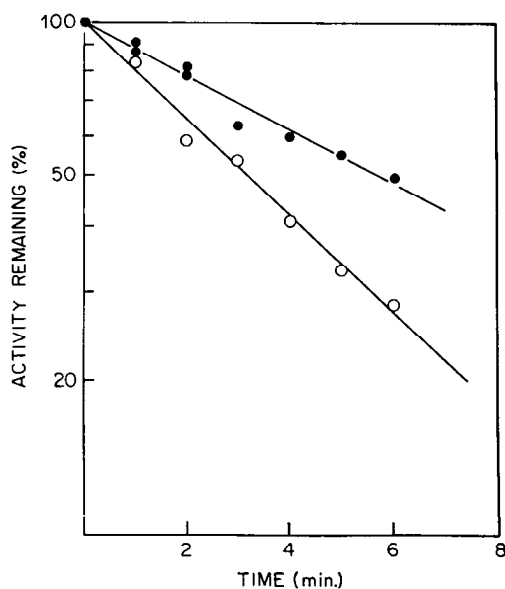


FIG. 3. Kinetic comparison of inhibition by deuterated and nondeuterated acetylenic thioesters. To cuvettes containing 0.5 ml of potassium phosphate, pH 6.0, ionic strength = 0.05, were added to a final concentration of  $8.0 \times 10^{-6}$  M, either 3-decynoyl-NAC (○) or 2,2-dideutero-3-decynoyl-NAC (●), and at zero time 33  $\mu$ g of dehydrase. At various times, 0.5 ml of  $3.1 \times 10^{-4}$  M *cis*-3-decynoyl-NAC in the same buffer was added and the initial velocity of product formation was recorded. The degree of inactivation was based on comparison with a similar reaction to which no acetylenic thioester had been added. Since the amount of acetylenic thioester was present at a 60-fold molar excess over dehydrase, the inactivation data were treated in a first order manner.

TABLE I

*Effects of acetylenic and allenic acids and thioesters on dehydrase activity*

Dehydrase was first incubated in 0.1 M potassium phosphate, pH 7.0, in the presence or absence of acid or thioester inhibitors. The thioester concentration in Experiment 1 was  $2 \times 10^{-6}$  M, and the incubation was carried out at 30° for 10 min; in Experiment 2 the acid concentration was  $1.6 \times 10^{-4}$  M and incubation lasted 30 min. Aliquots (10  $\mu$ l) were subsequently assayed with  $4 \times 10^{-4}$  M *cis*-3-decynoyl-NAC in 0.05 M Tris-HCl, pH 8.0, in a total volume of 1 ml.

Addition	$\Delta A_{263 \text{ nm}}$ per min	Control
		%
Experiment 1		
None.....	0.1014	100
3-Decynoyl-NAC.....	0.0030	3
2,3-Decadienoyl-NAC.....	0	0
Experiment 2		
None.....	0.0762	100
3-Decynoic acid.....	0.0803	105
2,3-Decadienoic acid.....	0.0206	26

lated. The latter possibility seemed more attractive in view of the enzyme's capacity to catalyze the transformation of *cis*-3-decynoyl-NAC to *trans*-2-decynoyl-NAC. An analogous enzymatic isomerization of the acetylenic thioester would afford the allenic NAC derivative, thus generating the true enzyme

TABLE II

*Amino acid composition of dehydrase treated with 3-decynoyl-NAC, 2,3-decadienoyl-NAC, and 2,3-decadienoic acid*

Dehydrase was inactivated with  $10^{-5}$  M 3-decynoyl-NAC,  $10^{-5}$  M 2,3-decadienoyl-NAC, or  $10^{-4}$  M 2,3-decadienoic acid and analyzed for amino acid content. A control contained untreated enzyme. Hydrolysis was for 23 hours. Those amino acids not listed were not determined.

Amino acid	Treatment				
	Acetylenic thioester	Allenic thioester	Allenic acid	Control	Previous work (2)
	<i>residues/18,000 g</i>				
Lysine.....	12.0	12.0	12.0	12.0	11.8
Histidine.....	1.2	1.3	1.4	1.9	2.2
Arginine.....	7.6	7.1	7.6	7.4	8.0
Aspartic acid.....	16.2	16.5	16.5	14.9	17.0
Threonine.....	9.1	8.6	8.1	7.8	7.3
Serine.....	10.1	6.2	5.6	6.0	4.0
Glutamic acid.....	13.4	14.0	14.6	13.8	14.2
Proline.....	7.8	7.0	8.4	8.3	7.0
Glycine.....	22.1	21.0	21.7	21.0	21.0
Alanine.....	9.6	11.2	11.4	9.2	11.7
Valine.....	8.8	7.8	8.7	8.3	13.4
Isoleucine.....	3.9	3.7	3.9	3.6	6.3
Leucine.....	16.4	16.0	15.4	16.9	17.6
Tyrosine.....	4.0	4.2	3.6	4.0	3.8
Phenylalanine.....	8.0	9.0	7.2	8.3	8.3

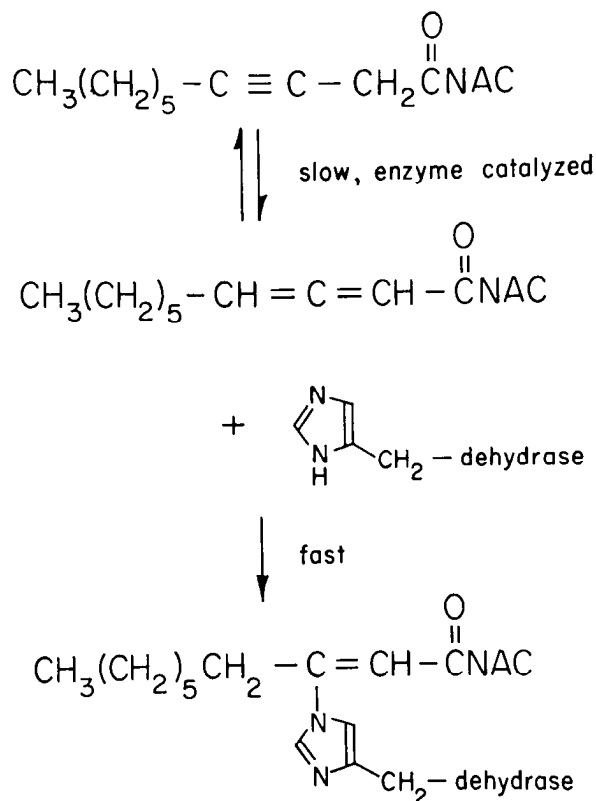
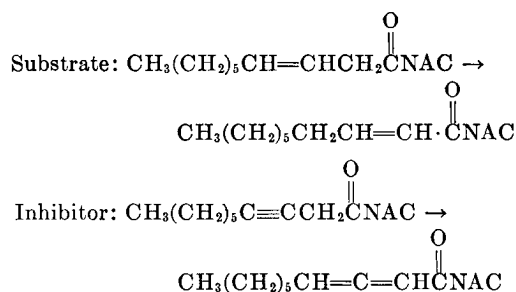


FIG. 4. Proposed mechanism for dehydrase inhibition by 3-decynoyl-NAC.

inhibitor:



To test this hypothesis, deuterium was incorporated into the  $\alpha$  position of the acetylene. A comparison of 3-decynoyl-NAC and 2,2-dideutero-3-decynoyl-NAC showed a marked difference in the time course of enzyme inhibition (Fig. 2A). Inactivation by the unlabeled acetylene was significantly more rapid than inactivation by the deuterated acetylene. When the kinetics of enzyme inactivation by the two compounds were examined, an average value of  $k_H:k_D = 2.05$  was found (Fig. 3). Correcting this figure for the isotopic composition of the deuterated molecule yielded a value of 2.60 for the ratio of the two rate constants. These experiments were performed at pH 6.0 in order to minimize nonenzymatic, base-catalyzed acetylene to allene conversion. Chemical isomerization was, in fact, not measurable at this pH. In an analogous study with the allenic thioesters, no isotopic effect was observed. 2-Deutero-2,3-decadienoyl-NAC inhibited dehydrase at an undiminished rate (Fig. 2B).

The kinetic isotope effect exhibited by the dideuterated acetylene and its absence with deuterated allene implicates removal of an  $\alpha$  proton as the rate-limiting step in the interaction between enzyme and 3-decynoyl-NAC. Loss of a proton at the  $\alpha$  position of the acetylene would, in fact, afford the thermodynamically more stable conjugated allene, and if enzyme-catalyzed, this process would be entirely analogous to olefinic isomerization. The rate differences of acetylene and allene inhibition, now substantiated by the kinetic isotope data, strongly support the notion that enzymatic isomerization is a requisite step in the mechanism by which 3-decynoyl-NAC inactivates dehydrase.

It is noteworthy that the magnitude of the above kinetic isotope effect is essentially the same as that observed for removal of  $\alpha$ -hydrogen in interactions of substrate with dehydrase. Thus, the observed  $k_H:k_D$  value for the dehydration of  $\beta$ -hydroxydecanoyl-NAC to *trans*-2-decenoyl-NAC was 2.25 (9).

Participation of the enzyme in the acetylene-allene conversion would explain why the thioester moiety, a grouping well known to facilitate hydrogen abstraction at  $\alpha$  carbons, is essential for the inhibitory action of 3-decynoyl-NAC. The same argument furthermore predicts that the thioester moiety may be less essential or even dispensable for enzyme inhibition by the isomerized product, *i.e.* once the facilitated removal of  $\alpha$ -hydrogen has occurred. Indeed, 2,3-decadienoic acid, in contrast to

3-decynoic acid, did inhibit dehydrase activity, albeit at very much higher concentrations than the NAC derivative (Table I). Inhibition by the allenic acid or by its NAC derivative could not be reversed by exhaustive dialysis. Free allenic acid inhibits most strongly at pH 5, less so at pH 7, and not noticeably at pH 9,<sup>3</sup> indicating that the protonated species is the active inhibitor. Assuming an acid dissociation constant of 4.5 to 5, this pH dependence would explain why relatively high concentrations of 2,3-decadienoic acid are needed for inhibition.

The action of 2,3-decadienoic acid and its thioester appears to be directed at the same target that is known to be sensitive to 3-decynoyl-NAC. Thus, enzyme exposed to the allenic reagents and subsequently hydrolyzed to its constituent amino acids contained 2 fewer histidyl residues than native dehydrase. Enzyme treated with 3-decynoyl-NAC showed the same reduction in histidine content (Table II).

Of some significance is the observation that 2,3-allenic acids and their derivatives, such as esters or nitriles, are highly susceptible to attack by a variety of nucleophiles (6, 10). In fact, the allenic isomers react much more readily than either 2- or 3-acetylenic derivatives (6). Thus, on the basis of comparative chemical reactivity alone, the allene is clearly the more plausible candidate as the direct enzyme inhibitor. The present experiments would then suggest that the interaction between 3-decynoyl-NAC and dehydrase may proceed as depicted in Fig. 4. The chemical structure of the derivative that is formed when allene reacts with enzyme histidine is currently under investigation.

The present experiments extend our previous understanding of the mode of action of the dehydrase inhibitor 3-decynoyl-NAC. They describe the rather unique case of an enzyme promoting its own destruction by catalyzing the transformation of a substrate analogue to an active site probe of extreme chemical reactivity.

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